APPENDIX B

Autologous Human Monocyte-Derived Dendritic Cells Genetically Modified to Express Melanoma Antigens Elicit Primary Cytotoxic T Cell Responses In Vitro: Enhancement by Cotransfection of Genes Encoding the Th1-Biasing Cytokines IL-12 and IFN-α[1]

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DNA-based immunization strategies designed to elicit cellular antitumor immunity offer an attractive alternative to protein- or peptide-based approaches. In the present study we have evaluated the feasibility of DNA vaccination for the induction of CTL reactivity to five different melanoma Ags in vitro. Cultured, monocyte-derived dendritic cells (DC) were transiently transfected with plasmid DNA encoding human MART-1/Melan-A, pMel-17/gp100, tyrosinase, MAGE-1, or MAGE-3 by particle bombardment and used to stimulate autologous PBMC responder T cells. CTL reactivity to these previously identified melanoma Ags was reproducibly generated after two or three stimulations with genetically modified DC. Co-ordinate transfection of two melanoma Ag cDNAs into DC promoted CTL responders capable of recognizing epitopes from both gene products. Coinsertion of genes encoding the Th1-biasing cytokines IL-12 or IFN-α consistently enhanced the magnitude of the resulting Ag-specific CTL reactivity. Importantly, DC transfected with a single melanoma Ag cDNA were capable of stimulating Ag-specific CTL reactivity restricted by multiple host MHC alleles, some of which had not been previously identified. These results support the inherent strengths of gene-based vaccine approaches that do not require prior knowledge of responder MHC haplotypes or of relevant MHC-restricted peptide epitopes. Given previous observations of in situ tumor HLA allele-loss variants, DC gene vaccine strategies may elicit a greater diversity of host therapeutic immunity, thereby enhancing the clinical utility and success of such approaches. *The Journal of Immunology*, 1998, 160: 1139–1147.

Recent observations that protective antiviral immunity can be induced following genetic immunization with plasmid DNA encoding viral Ags (1–4) have suggested novel approaches for vaccine construction. Direct inoculation of naked DNA into the skin or muscle of animals results in both humoral and cellular immune responses. Such immunization strategies promise important advantages over protein- or peptide-based protocols. Gene expression in host cells leads to the endogenous processing and presentation of antigenic peptides by some or all self-MHC alleles. Importantly, specific T cell-mediated immunity may be stimulated by vaccine-involved APC without prior knowledge of responder MHC haplotypes or of relevant MHC class I- or class II-restricted peptide epitopes. Several genes encoding Ags of interest can be applied simultaneously, including genes encoding immunostimulatory cytokines such as GM-CSF[3] and IL-12, which have been shown to direct the nature of the resulting immune response and to augment the efficacy of DNA-based vaccines (5–8).

With the molecular identification of melanoma Ags recognized by CTL (9), there has been increasing interest to apply gene-based strategies in the development of melanoma vaccines (10). The potencies of vaccines employing plasmid DNA or recombinant viral vectors encoding model tumor Ags have been confirmed in rodent models (11–18). Condon et al. (18) reported that particle bombardment of the skin, delivering cDNA encoding chicken OVA as a model tumor Ag, resulted in the induction of Ag-specific CTL capable of mediating protective antitumor immunity against an OVA-transfected B16 mouse melanoma. Interestingly, direct transfection of skin-derived dendritic cells (DC) was demonstrated, with some of these cells ultimately localizing in the draining lymph nodes. Using bone marrow chimeric mice, several other groups have shown that the induction of CTL responses following plasmid DNA vaccination depends on the presentation of the encoded Ag by bone marrow-derived APC (19, 20). DC are believed to be essential for the induction of primary, cell-mediated immune responses (21, 22). It is therefore hypothesized that DC acquire

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[3] Abbreviations used in this paper: GM-CSF, granulocyte-macrophage colony-stimulating factor; DC, dendritic cells; h, human; PE, phycoerythrin; RLU, relative light units.

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gene products in situ following DNA vaccination, either through direct transfection or indirectly via phagocytotic or macropinocytotic mechanisms from other transfected cells. They then migrate to locoregional lymphoid organs, where the processed Ag is presented to responding T cells.

In this study, we have evaluated the potential of DNA vaccination for immunotherapy of malignant melanoma in an in vitro vaccine model. We have chosen particle bombardment to target expression plasmids encoding the human melanoma Ags MART-1/Melan-A, pMel-17/gp100, tyrosinase, MAGE-1, or MAGE-3 into monocyte-derived DC. Transfected DC were used as stimulators to prime Ag-specific CTL responses in vitro. Our main goal was to assess whether primary CTL responses against several different melanoma Ags can be obtained with multiple HLA restriction elements when using plasmid DNA as an immunogen. Further, we investigated whether cotransfection of cytokine expression plasmids encoding IL-12 or IFN-α would augment the resultant Ag-specific cytolytic T cell responses.

Materials and Methods

Cell lines

The human melanoma cell lines Mel-397 (HLA-A1$^+$, A10$^+$, B8$^+$, B62$^+$, MART-1/Melan-A$^+$, gp100$^+$, MAGE1$^+$, MAGE3$^+$), Mel-526 (HLA-A2$^+$, A3$^+$, B50$^+$, B62$^+$, MART-1/Melan-A$^+$, gp100$^+$, tyrosinase$^+$, MAGE1$^+$, MAGE3$^+$), and Mel-624 (HLA-A2$^+$, A3$^+$, B7$^+$, B14$^+$, MART-1/Melan-A$^+$, gp100$^+$, tyrosinase$^+$, MAGE1$^+$, MAGE3$^+$) were provided by Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD). The EBV-B cell lines CIR.A2 and CIR.A3 were described previously (23). The EBV-B cell line CW-EBV (A1$^+$, A31$^+$, B8$^+$, B35$^+$) was generated using standard methods (24) from healthy donor PBMC. Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and were determined to be free of *Mycoplasma* contamination (GeneProbe, Fisher Scientific, Pittsburgh, PA). All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Antibodies

Supernatants containing mAb produced by the following hybridomas, which were obtained from the American Type Culture Collection (Rockville, MD), were used in this study: anti-HLA class I monomorphic (W6/32, HB-95), anti-HLA-A2 (MA2.1, HB-54), anti-HLA-A3 (GAP-A3, HB-122), and anti-human Ia-like molecules (L243, HB55).

Peptides

Peptides were synthesized by standard F-moc chemistry and purified by reverse phase HPLC in the Peptide Synthesis Facility of the University of Pittsburgh Cancer Institute (Shared Resource). The following sequences were synthesized: MART-1/Melan-A_{27-35} (AAGIGILTV), MART-1/Melan-A_{32-40} (ILTVILGVL), pMel-17/$gp100_{17-25}$ (ALLAVGATK), pMel-17/$gp100_{280-288}$ (YLEPGPVTA), $tyrosinase_{1-9}$ (MLLAVLYCL), $tyrosinase_{368-376}$ (YMDGTMSQV), MAGE-$1_{161-169}$ (EADPTGHSY), MAGE-$3_{161-169}$ (EVDPIGHLY), and MAGE-$3_{271-279}$ (FLWGPRALV). Purity exceeded 90% based on mass spectrometry for m.w. and by mass spectrometry/mass spectrometry fragmentation, providing unambiguous sequencing data (University of Pittsburgh Biotechnology Center, Mass Spectrometry Facility).

Plasmid DNA

The plasmid pCMV-lux containing a CMV-driven firefly luciferase gene was provided by Geniva (Madison, WI). The expression plasmid pCI using the CMV immediate-early gene promoter and a chimeric intron was purchased from Promega (Madison, WI). The expression plasmid pCMV-A using the CMV immediate-early gene promoter and intron A was provided by Geniva. The plasmid pCMV-A-hIFN-α2b was constructed by ligating a *Not*I-*Eco*RI fragment containing the hIFNα-2b cDNA (provided by Schering Plough Research Institute, Kenilworth, NJ) into CMV-A. The pCMV-A-hIL-12 (p40-IRES-p35) was constructed by ligating a *Bam*HI fragment containing the IRES sequence from EMCV followed by the human p35 cDNA (25) into pCMV-A-p40 (provided by Geniva). The pCI-MART-1 was constructed by ligating a *Sal*I-*Not*I fragment containing the hMART-1 cDNA (provided by Dr. M. Mäurer) into pCI. The pCI-pMel-17 was constructed by ligating a *Sal*I-*Not*I fragment containing the human pMel-17 cDNA (26) into pCI. The pCI-tyrosinase was constructed by ligating an *Eco*RI fragment containing the human tyrosinase cDNA (27) into pCI. The pcDSRα-MAGE1 (28) and pcDSRα-MAGE-3 (29) use the SRα promoter, which is composed of the SV40 early promoter followed by the R segment and part of the U5 sequence of the long terminal repeat of human T cell leukemia virus type 1. All vectors express only the Ag or cytokine of interest under a eukaryotic promoter. Plasmids were grown in *Escherichia coli* strain DH5α and purified using Qiagen Endofree Plasmid Maxi Kits (Qiagen, Chatsworth, CA).

Generation of DC

DC were prepared from PBMC as previously described (30). Briefly, PBMC were isolated from leukapheresed blood of healthy donors by density centrifugation on Ficoll-Hypaque gradients (1.077 g/ml; LSM, Organon-Teknika, Durham, NC) for 20 min at 2000 rpm at room temperature. After four or five washes in HBSS (Life Technologies, Gaithersburg, MD) to remove platelets, cells were resuspended at 10^7/ml in AIM-V medium (Life Technologies) and incubated for 1 h in 75-cm^2 tissue culture flasks (37°C, 5% CO_2). Nonadherent cells were gently washed out with HBSS and cryopreserved for use as T cell responders. The remaining plastic-adherent cells were further cultured (37°C, 5% CO_2) in AIM-V medium supplemented with 1000 U/ml rGM-CSF and 1000 U/ml rIL4 (Schering-Plough). After 7 to 10 days, nonadherent cells were harvested. DC generated in this way were 50 to 80% pure based on morphology and the expression of a CD3$^-$, CD14$^-$, CD16$^-$, CD20$^-$, CD40$^+$, CD80$^+$, CD86$^+$, MHC class II$^+$ immunophenotype as assessed by flow cytometry. In some experiments DC were purified further (>95%) by density gradient centrifugation. No significant difference in CTL inductions was noted when using irradiated DC stimulators within the 50 to 95% range of purity.

Flow cytometry

For immunophenotyping, DC or T cell responders were washed in HBSS supplemented with 1% BSA and 0.1% NaN_3 and incubated (30 min at 4°C) with one of the following mAb: PE-conjugated anti-HLA-DR (Becton Dickinson, Mountain View, CA), FITC-conjugated anti-CD80 (Ancell, Bayport, MN), FITC-conjugated anti-CD86 (PharMingen, San Diego, CA), FITC-conjugated anti-CD40 (PharMingen), PE-conjugated anti-CD3 (Becton Dickinson), FITC-conjugated anti-CD4 (Becton Dickinson), PE-conjugated anti-CD8 (Becton Dickinson), FITC-conjugated anti-CD14 (Becton Dickinson), PE-conjugated anti-CD16 (Becton Dickinson), and FITC-conjugated anti-CD20 (Becton Dickinson). DC were also stained with corresponding isotype-matched control mAb (PharMingen). Surface expression was analyzed using a FACScan flow cytometer (Becton Dickinson) and LYSIS II software; data were collected on 5,000 to 10,000 viable cells.

Particle-mediated gene transfer to DC

Plasmid DNA was precipitated onto 2.6-μm gold particles at a density of 2 μg of DNA/mg of particles as previously described (14, 18). Briefly, gold particles and DNA were resuspended in 100 μl of 0.05 M spermidine (Sigma Chemical Co., St. Louis, MO), and DNA was precipitated by the addition of 100 μl of 1 M $CaCl_2$. Particles were washed in dry ethanol to remove H_2O, resuspended in dry ethanol containing 0.075 mg/ml of polyvinylpyrrolidone (Sigma Chemical Co.), and coated onto the inner surface of Tefzel tubing using a tube loader. The tubing was cut into 0.5-in. segments, resulting in the delivery of 0.5 mg of gold coated with 1 μg of plasmid DNA/transfection with the Accell helium pulse gun. Gold particles, tubing, tube loader, and the Accell helium pulse gun were provided by Auragen/Geniva (Middleton, WI). Monocyte-derived DC were transfected in suspension in six-well plates. DC were harvested and pelleted by centrifugation; 2×10^6 cells were resuspended in 20 μl of fresh medium and spread evenly in the center of a well. Cells were bombarded at a pressure of 300 psi of helium, and fresh culture medium was added immediately. Five to ten percent of DC can be transfected, as assessed using enhanced green fluorescent protein (pEGFP, Clontech, Palo Alto, CA) as a reporter gene.

Assays for expression of luciferase

Expression of luciferase was determined 8 to 72 h after gene transfer to DC. Cells were washed with HBSS (Life Technologies), lysed in 100 μl of cell culture lysis reagent (Promega), and stored at −80°C. Samples were thawed, cell debris was pelleted, and 10 μl of cell extract was assayed in duplicate with the Luciferase Assay System (Promega) using an Autolumat LB 953 (eG&G Berthold) set to integrate emission data over 10 s. The level

of sample luminescence was recorded as relative light units (RLU). Experiments were performed at least three times. Using recombinant luciferase protein (Promega), 1 pg was measured as 600 RLU.

ELISA for human IFN-α2b and IL-12

Reagents for sandwich ELISAs were provided by Dr. P. Zavodny (Schering Plough Research Institute) for IFN-α and by Dr. M. Gately (Hoffmann LaRoche, Nutley, NJ) for IL-12. The following Abs were coated onto 96-well microtiter ELISA plates (Immulon 2, Dynatech Laboratories, Chantilly, VA) at a concentration of 5 μg/ml in 100 mM sodium carbonate buffer, pH 9.5 (16 h at 4°C), for cytokine capture: a polyclonal sheep anti-human IFN-α Ab (Schering Plough) or a monoclonal mouse anti-human p40 Ab (clone 20C2, Hoffmann-La Roche). Nonspecific binding was blocked with 3% (w/v) BSA, 0.01% thimerosal (w/v), and 0.05% (v/v) Tween-20 in PBS (8–24 h at 4°C) followed by incubation of samples along with half-log dilutions of recombinant hIFN-α2b (Schering Plough) or hIL-12 (Hoffmann-La Roche) in PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween-20 (16 h at 4°C). The following Abs were used at a concentration of 1 μg/ml in PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween-20 (2 h at room temperature) for detection: a monoclonal mouse anti-human IFN-α2 Ab (clone NK2, Schering Plough) followed by a peroxidase-conjugated goat anti-mouse IgG (1/20,000; Jackson ImmunoResearch Laboratories, West Grove, PA) or a peroxidase-conjugated monoclonal mouse anti-human p70 Ab (clone 4D6, Hoffmann-La Roche). The TMB microwell peroxidase substrate system (Kirkegaard and Perry, Gaithersburg, MD) was used for color development, the reaction was stopped with 1 N H_2SO_4, and plates were read at 450 nm with a Dynatech MR500 ELISA plate reader. The lower limits of detection were 1 U/ml for hIFN-α2b and 30 pg/ml for hIL-12.

Induction of CTL using DC genetically engineered to express melanoma Ags

DC were irradiated (3000 rad) and transfected by particle bombardment. Cryopreserved nonadherent PBMC were used as responders. CTL priming cultures were set up in six-well plates by mixing 10^6 autologous DC with 2 to 5 × 10^7 nonadherent responder PBMC in 5 ml of AIM-V medium supplemented with 0.25 ng/ml rhIL-1, 0.25 ng/ml rhIL-7 (kindly provided by Immunex Corp., Seattle, WA), and 5% human AB serum (Sigma). Responding T cells were restimulated weekly using irradiated (3000 rad), transfected, autologous DC at a 20:1 responder to stimulator ratio in AIM-V medium containing 10 IU/ml of IL-2 (Chiron, Emeryville, CA).

Cytotoxicity assays

CTL effectors were tested for their cytolytic reactivity after two or three restimulations against melanoma cell lines or peptide-pulsed EBV-B cells in duplicate in standard 4-h ^{51}Cr release assays using 96-well round-bottom plates. Target cells (2 × 10^6) were radiolabeled with 100 μCi of Na_2-$^{51}CrO_4$ (New England Nuclear-DuPont, Bedford, MA) for 1 h at 37°C. Peptide-pulsed targets were prepared by incubating cells with peptide at a concentration of 1 μg/ml for 1 h at 37°C before and 0.5 μg/ml during the cytotoxicity assay. For Ab blocking, targets were incubated with hybridoma supernatant 30 min before and during the assay at a final dilution of 1/5. For $CD4^+$ T cell depletions, anti-CD4-coupled magnetic beads were used according to the manufacturer's instructions (Dynal, Oslo, Norway). The percentage of specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Target cells incubated in medium alone or in medium containing 5% Triton X-100 (Sigma Chemical Co.) were used to determine spontaneous and maximum ^{51}Cr release, respectively. Counts generated in the cytotoxicity assays usually averaged 100 to 200 cpm for spontaneous and 1500 to 3000 cpm for maximum release, with spontaneous release <15% of maximum release in any given assay.

Cytokine release assays

A long term CTL line recognizing the MART-1/Melan-A_{27-35} peptide was used as a source of responder cells. CTL were washed twice before use. Cytokine release assays were performed in duplicate using 96-well round-bottom plates with 2.5 × 10^4 peptide-pulsed or 10^5 tumor-associated Ag-transduced DC as stimulators and 2.5 × 10^4 CTL as responders in complete medium. Peptide-pulsed DC were prepared by incubation with 1 μg/ml peptide for 1 h at 37°C and washed twice in HBSS. Supernatants were harvested after 24 h, and the hIFN-γ content was measured using an Endogen cytokine ELISA kit (Woburn, MA). The lower limit of detection was 30 pg/ml.

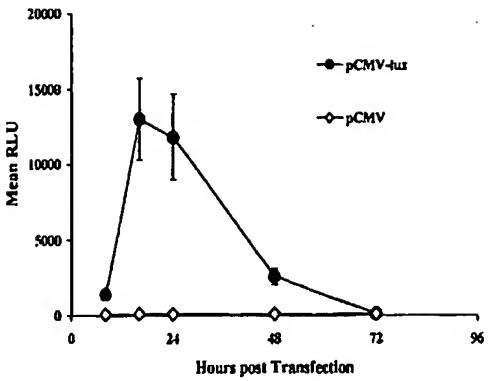

FIGURE 1. Transient expression of the marker gene firefly luciferase after particle-mediated gene transfer into PBMC-derived cultured human DC. Day 7 cultured DC were transfected with expression plasmids encoding firefly luciferase (pCMV-lux) or vector control (pCMV) using the gene gun. DC were lysed 8 to 72 h following gene transfer, and expression of luciferase per 10^5 cells was measured using a luminometer. Results are expressed as the mean RLU ± SEM and show transient transfection of DC with maximal levels of sample bioluminescence being detected 16 to 24 h after transfection.

Results

Expression of firefly luciferase following particle-mediated gene transfer to DC

A firefly luciferase gene was used as a sensitive reporter gene to optimize the parameters for particle-mediated gene transfer to DC using the Accell helium pulse gun. Expression of luciferase was monitored over an 8- to 72-h time course (Fig. 1). Significant levels of transgene were detected within 8 h, with maximal levels occurring 16 to 24 h after transfection. DC transfected with an irrelevant plasmid (pCI) produced only background levels of bioluminescence. Bombardment of 2 × 10^6 DC with 1 μg of plasmid DNA coated onto 0.5 mg of gold particles delivered at a pressure of 300 psi of helium resulted in the highest transgene expression while maintaining >75% of the cell viability vs mock transfected cultures. At 24 h post-transfection, about 12,000 RLU (corresponding to 20 pg) was detected per 10^5 cells.

Expression of IL-12 and IFN-α following particle-mediated gene transfer to DC and effect of transfected cytokine on DC phenotype

DC cultures were transfected with expression plasmids encoding hIL-12 or hIFN-α2b, and production levels were measured in culture supernatants by ELISA after 48 h. Transfected DC regularly produced 40 to 200 pg of hIL-12 and 10 to 50 U of hIFN-α2b/10^6 cells, respectively. Comparable levels of cytokine production were observed following transfection with cytokine cDNA alone or in combination with tumor Ag cDNA. Supernatants from IL-12 transfectants contained <5 U of hIFN-α2b, and supernatants from IFN-α transfectants contained <50 pg of hIL-12 (Table I).

Transfection of both hIL-12 and hIFN-α2b resulted in a more immunostimulatory phenotype of DC as assessed by flow cytometry 48 h after transfection. The expression intensities of MHC class II and the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) were increased relative to those of control transfected DC, especially following IFN-α transfection (Table I). Similar phenotypic changes were observed following DC treatment with the respective recombinant cytokines (data not shown).

Table I. *Level of production and effect of transfected cytokine cDNA on DC phenotype*[a]

cDNA Transfected	Cytokine Production/ 10^6 Cells/24 h	Immunophenotype (MFC) CD80	CD86	MHC II
pCMV-A	<5 U IFN-α2b; <30 pg IL-12	185	58	50
pCMV-A-hIFN-α2b	10–50 U IFN-α2b	409	255	82
pCMV-A-hIL-12	50–200 pg IL-12	222	184	54

[a] Cultured human DC were transfected with the indicated plasmids using particle-mediated gene transfer as outlined in *Materials and Methods*. Transfected cells were then cultured at 37°C. One-half of the culture supernatant was aspirated for ELISA evaluation at 24 h and replaced with fresh culture medium. DCs were harvested at 48 h and phenotyped for expression of the costimulatory molecules CD80 (B7.1), CD86 (B7.2), and MHC class II. Results are reflective of four such experiments performed.

Table II. *Summary of donor CTL responses induced with autologous DC genetically engineered to express melanoma Ags*[a]

DC Transfected with	No. of Responders/ Total	CTL Restricted by
MART-1/Melan-A	3/3	HLA-A2, -A3
Pmel17/gp100	5/5	HLA-A1, -A2, -A3, -B8
Tyrosinase	1/3	HLA-A2, -A3
MAGE-1	7/7	HLA-A1, -A2, -A3
MAGE-3	6/6	HLA-A1, -A2, -A3, -B8

[a] A total of 10 donors were evaluated for their CTL responses after three in vitro stimulations with autologous DC genetically engineered to express melanoma Ags. The HLA-matched allogeneic melanoma cells Mel 397 (HLA-A1, -A10), Mel 526 (HLA-A2, -A3), and Mel 624 (HLA-A2, A3) expressing all of the above Ags were used as targets in standard ^{51}Cr release assays. CTL reactivity of responders ranged from 16 to 58%, with >80% inhibition by the relevant anti-HLA class I monomorphic mAb W6/32, anti-HLA-A2 mAb MA2.1, or anti-HLA-A3 mAb GAP-A3, but not by mAb L243. Bold entries are restriction elements that appear to contain novel CTL reactivity. The donor HLA-A haplotypes were: D_1: A2, -; D_2: A1, A3; D_3: A2, -; D_4: A2, A3; D_5: A2, -; D_6: A3, -; D_7: A1, A3, B8; D_8: A1, A3; D_9: A2, -; D_{10}: A2, -.

DC transfected with MART-1/Melan-A stimulate a peptide-specific CTL line

The pCI-MART-1 was transfected into HLA-A2$^+$ cultured DC by particle-mediated gene transfer. Twenty-four hours later these DC were assessed for their ability to stimulate cytokine release from an HLA-A2-restricted CTL line recognizing the MART-1/Melan-A_{27-35} peptide epitope. The pCI-MART-1-transfected DC stimulated significantly higher levels of IFN-γ release than pCI backbone-transfected controls (1800 ± 450 vs 260 ± 80 pg/ml/24 h). Transfected DC or CTL alone did not secrete detectable amounts of IFN-γ (<30 pg/ml/24 h). This demonstrates that transduced DC expressed, processed, and presented T cell epitopes derived from MART-1/Melan-A in an immunogenic format.

Autologous DC transfected with five different melanoma Ag cDNAs elicit Ag- and tumor-reactive CTL in vitro

DC were generated from healthy, HLA-A1$^+$, HLA-A2$^+$, and/or HLA-A3$^+$ donors; gene gun-transfected with expression plasmids encoding the melanoma Ags MART-1/Melan-A, pMel-17/gp100, tyrosinase, MAGE-1, or MAGE-3, irradiated (3000 rad); and used to stimulate autologous responder T cells as described in *Materials and Methods*. Restimulations with autologous, transfected DC were performed 7 and 14 days later, and the CTL reactivity of bulk cultures was assessed on day 21. The melanoma cell lines Mel-397 (HLA-A1$^+$, HLA-A10$^+$, MART-1/Melan-A$^+$, gp100$^+$, MAGE1$^+$, MAGE3$^+$), Mel-526 (HLA-A2$^+$, A3$^+$, MART-1/Melan-A$^+$, gp100$^+$, tyrosinase$^+$, MAGE1$^+$, MAGE3$^+$), and Mel-624 (HLA-A2$^+$, A3$^+$, MART-1/Melan-A$^+$, gp100$^+$, tyrosinase$^+$, MAGE1$^+$, MAGE3$^+$) were used to determine reactivity against tumor cells that constitutively express the immunizing Ag. Reproducible induction of CTL reactivity to these HLA-matched allogeneic melanomas was observed using all five melanoma Ag cDNAs. HLA restriction was confirmed using the blocking mAb W6/32 (anti-HLA class I monomorphic), MA2.1 (anti-HLA-A2), or GAP A3 (anti-HLA-A3). The mAb L243 (anti-human Ia-like molecules) was used as a control. A summary of these experiments is shown in Table II.

The Ag specificity of responses was monitored using the EBV-B cell lines CW-EBV (A1$^+$, A31$^+$), C1R.A2 (A2$^+$, $^-$), and C1R.A3 (A3$^+$, $^-$) pulsed with peptides previously identified as CTL epitopes derived from the respective gene products. We frequently observed only low levels of cytotoxic reactivity directed against a given peptide, while an HLA-matched melanoma was lysed to a significantly higher degree. However, this may merely reflect the reactivity of bulk CTL against multiple epitopes co-ordinately processed and presented from the immunizing melanoma-associated gene product, only one of which we analyzed. Importantly, we noted cytotoxic reactivity only against peptide products derived from the gene used to prime the respective CTL cultures, but never against peptide products derived from unrelated control genes. CTL responses from two representative HLA-A2$^+$ donors are illustrated in Figure 2.

Co-ordinate transfection of two melanoma Ag cDNAs into autologous DC induces Ag-specific CTL reactivity to peptides derived from both gene products

The possibility of administering multiple immunogenic Ags simultaneously into DC was investigated in subsequent experiments. Two different plasmids encoding melanoma Ags were cotransfected into cultured DC and used to stimulate autologous PBMC responders using the in vitro induction protocol as outlined above. We reproducibly could generate Ag-specific CTL reactivity against target cells pulsed with peptides derived from both gene products, which could be blocked by the addition of mAb W6/32 but not that of mAb L243. Again, no cytotoxicity was observed against targets pulsed with irrelevant peptides. A representative experiment of four is depicted in Figure 3.

Coinsertion of genes encoding IL-12 or IFN-α enhances the induction of tumor-reactive CTL in vitro

We next evaluated whether cotransfection of plasmids encoding immunostimulatory cytokines along with tumor Ag cDNA into autologous DC would enhance the resultant cellular immune response. IL-12 and IFN-α were chosen due to their reported abilities to enhance Th1-associated immune responses. Using the gene gun, donor DC were transfected with tumor Ag cDNA alone, tumor Ag cDNA plus IL-12 cDNA (p35-IRES-p40), or tumor Ag cDNA plus IFN-α2b cDNA. After irradiation (3000 rad), these gene-modified DC were used as primary in vitro stimulators for autologous responder PBMC-T cells. Bulk cultures were restimulated at 7 and 14 days with irradiated autologous DC transfected with the relevant tumor Ag cDNA alone. Bulk responder cultures harvested on day 21 (i.e., 7 days after the third weekly stimulation) displayed cytokine-dependent changes in T cell yield and phenotype. Cultures induced without cytokine cDNA cotransfection contained typically 15 to 35% CD8$^+$ T and 54 to 72% CD4$^+$ cells, with an average CD8/CD4 ratio of 0.5 ± 0.2. Cultures induced by IL-12-transfected DC contained 71 ± 21% more T cells, with a significantly higher CD8/CD4 ratio (0.9 ± 0.2). Cultures induced with IFN-α-transfected DC also contained more T cells (47 ± 22%) than controls; however, the CD8/CD4 ratio was slightly reduced (0.4 ± 0.1) compared with that of the control group. The

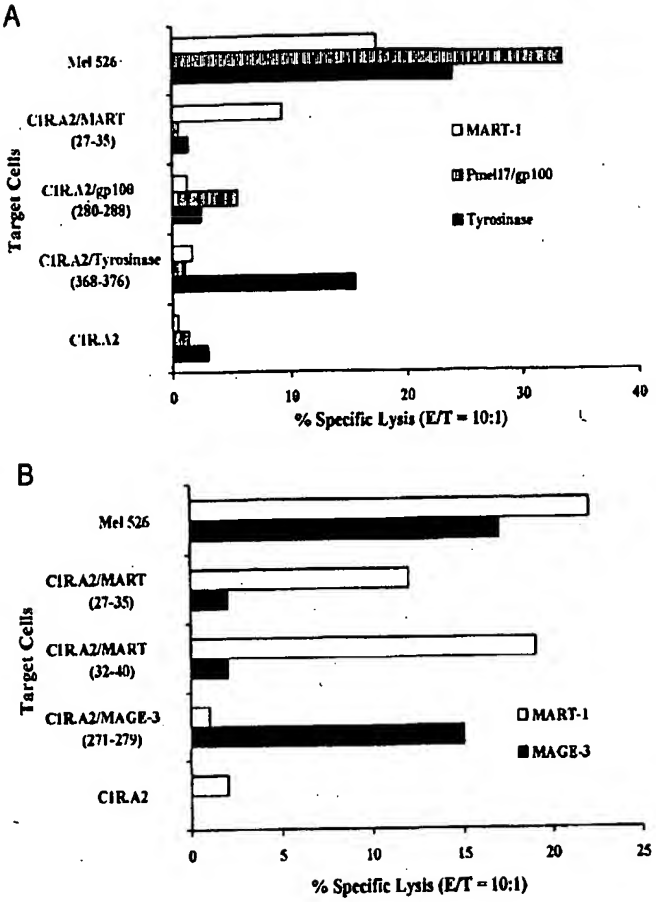

FIGURE 2. HLA-A2$^+$ donor DC transfected with melanoma Ag cDNA elicit Ag-specific and tumor-reactive CTL in vitro. HLA-A2$^+$ (A29$^+$, B7$^+$, B44$^+$, Cw2$^+$, Cw7$^+$; *A*) or HLA-A2$^+$ (A1$^+$, B7$^+$, Cw4$^+$, Cw7$^+$; *B*) monocyte-derived DC were generated in 7-day cultures as outlined in *Materials and Methods*; transfected with plasmids encoding MART-1/Melan-A, pMel-17/gp100, tyrosinase, MAGE-1, or MAGE-3 (legends); irradiated (3000 rad); and used to stimulate autologous responder PBMC-T cells on a weekly basis. Seven days after the third stimulation, CTL reactivities were assessed in 4-h cytotoxicity assays against Mel-526 (expressing all Ags) or the C1R.A2 EBV-B cell line prepulsed with the indicated HLA-A2-binding synthetic peptides. These data are representative of responses observed for a total of six HLA-A2$^+$ donors evaluated.

Ag-specific, HLA class I-restricted CTL reactivity was always greater in either of the cytokine-transfected groups than in the control group, although the rank order of efficacy of these two cytokines in enhancing CTL reactivity did vary between individuals and, in certain instances, between specific responses against different Ags in the same individual. A representative experiment is illustrated in Figure 4.

Identification of novel MHC-restricted CTL reactivities against gp100 and MAGE-3 using in vitro gene vaccination

We evaluated the ability of tumor Ag cDNA with or without cytokine cDNA-transfected DC to stimulate CTL cultures exhibiting tumor-specific cytolytic reactivity restricted by multiple HLA class I alleles. A representative experiment is displayed in Figure 5 for

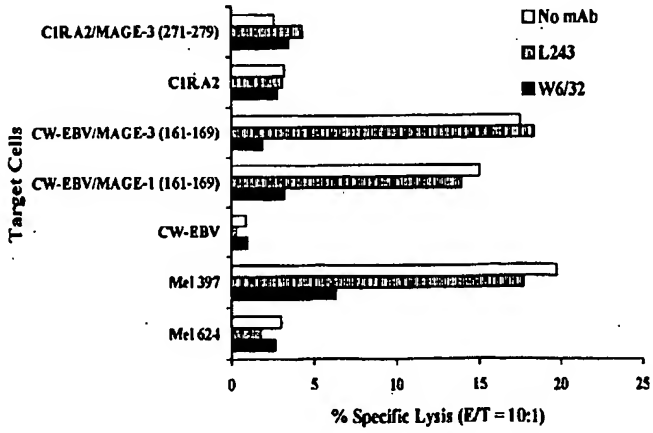

FIGURE 3. DC cotransfected with both MAGE-1 and MAGE-3 cDNAs elicit CTL responders capable of recognizing epitopes from both tumor Ags. Autologous cultured DC cotransfected with MAGE-1 and MAGE-3 cDNA were used three times to stimulate PBMC-T cell responders from an HLA-A1$^+$, -A2$^+$ (-B7$^+$, -B39$^+$, -Cw7$^+$) donor. HLA-restricted cytolytic reactivity could be detected against Mel-397 (A1$^+$, A10$^+$), but not Mel-624 (A2$^+$, A3$^+$), and against CW-EBV (A1$^+$, A31$^+$) pulsed with the MAGE-1$_{161-169}$ as well as the MAGE-3$_{161-169}$ peptide, but not against unpulsed CW-EBV. The class I-restricted nature of CTL reactivity was verified by addition of the anti-class I monomorphic mAb W6/32. The anti-class II mAb L243 served as an isotype-matched control. In this donor, no reactivity was observed against C1R.A2 cells pulsed with the previously identified HLA-A2-presented MAGE-3$_{271-279}$ peptide. In two of three alternate experiments involving HLA-A2$^+$ donors, however, CTL reactive to MAGE-3$_{271-279}$ were identified.

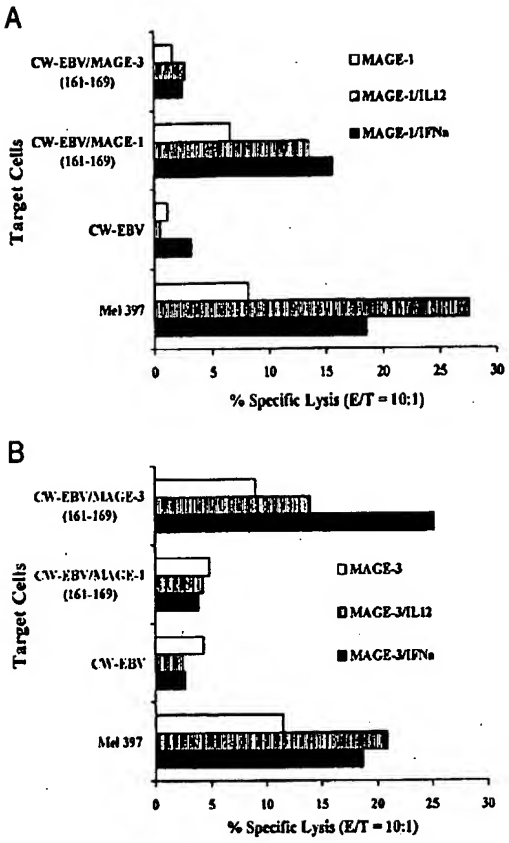

FIGURE 4. IL-12 or IFN-α cDNA transfection enhance tumor Ag gene induction of tumor-reactive CTL in vitro. Cultured DC obtained from an HLA-A1$^+$ (A3$^+$, B7$^+$, B35$^+$, Cw4$^+$, Cw7$^+$) donor were transfected with cDNA encoding either MAGE-1 (*A*) or MAGE-3 (*B*) with or without cDNA encoding hIL-12 or hIFN-α2b, irradiated, and used to restimulate autologous PBMC-T cell responders. Restimulations were performed twice with autologous DC transfected with tumor Ag only, i.e., cytokine cDNA was present only in the primary in vitro stimulation. CTL were assessed 7 days after the third stimulation. Targets included the MAGE-1$^+$, MAGE-3$^+$ Mel-397 and the HLA-A1$^+$ EBV-B cell line CW-EBV with or without synthetic peptide. Data are representative of responses observed for 10 donors evaluated.

an HLA-A1$^+$, -A3$^+$, -B8$^+$, -B51$^+$, -Cw3$^+$ responder. HLA-A1- or B-8-restricted CTL reactivity was identified for both gp100/pMel-17- and MAGE-3-induced cultures and was enhanced by the presence of IL-12 or IFN-α cotransfection (Fig. 5*A*). HLA-A3-restricted CTL reactivity was also identified in these cultures, again enhanced by cytokine cotransfection (Fig. 5*B*). This reactivity was significantly inhibited by the anti-HLA-A3-specific mAb GAP-A3 and was at least partially specific for the recently identified $gp100_{17-25}$ epitope (31).

Interestingly, both B cell line targets (CW-EBV and CIR.A3) used in this experiment displayed significant constitutive sensitivity to MAGE-3-induced CTL, particularly in those cultures involving cytokine cotransfection. A PCR screen of CIR.A3 supports the expression of the MAGE-3 gene product. This is consistent with reports that EBV-B cell lines occasionally express genes of the MAGE family (32). We are currently performing a more detailed study, including immunologic evaluation of our EBV-B cell line panel.

In summary, our experiments demonstrate that Ag-specific CTL reactivity can be elicited in vitro against the five different melanoma Ags studied using naked plasmid DNA transfected into cultured DC as stimulators. These and analogous experiments involving different donors (data not shown) support the existence of naturally processed and shared HLA-A1-, HLA-A2-, and HLA-A3-presented epitopes derived from the MART-1, gp100/pMel-17, tyrosinase, MAGE-1, and MAGE-3 gene products.

Discussion

Culture techniques allowing for the generation of large numbers of immunostimulatory DC in vitro (33–35) have stimulated significant interest in the use of DC as a biologic adjuvant for DNA vaccines (36–42). Here we show the induction of primary, Ag-specific CTL responses in vitro with naked plasmid DNA encoding melanoma Ags transfected into cultured autologous DC. Importantly, we present data for five different melanoma Ags: MART-1/Melan-A, pMel-17/gp100, tyrosinase, MAGE-1, and MAGE-3. MHC-restricted CTL responses were reproducibly induced against HLA-matched allogeneic melanomas. Ag specificitywas confirmed using HLA-matched EBV-B cell lines pulsed with peptides derived from the respective gene products. While the observed levels of Ag-specific lysis were generally low, they are consistent with the early stage of the culture (i.e., third restimulation only) and the minority of CD8$^+$ T cells found in the bulk cultures (15–35% CD8$^+$). We have since demonstrated higher lytic values when CD4$^+$ cells are depleted from the cultures or upon extended in vitro restimulations (Fig. 6).

Although the use of viral vectors may be more efficient for gene transfer into DC (40, 42), nonviral gene delivery methods have several important advantages of potential clinical interest. 1) More than one gene can readily be transfected simultaneously, allowing for cotransfection of genes encoding distinct tumor Ags and/or immunostimulatory cytokines. 2) Only the gene of interest is transcribed without immunologic interference from viral proteins both in vitro and in vivo (leading to neutralizing Abs that can limit effective repeated application of recombinant viruses). 3) There is no risk of recombination associated with replication-deficient viral vectors. 4) Insertion of foreign DNA into the genome is less likely due to the transient nature of gene transfer. 5) The approach uses highly purified DNA that can readily be produced in large quantities and is very stable.

Our approach allowed us to perform a preliminary assessment of the combination of tumor Ag cDNA with cytokine cDNA on the induction of primary CTL responses in vitro. We observed that cotransfection of plasmids encoding the immunostimulatory cytokines IL-12 or IFN-α along with tumor Ag cDNA consistently enhanced Ag-specific CTL induction. This enhancement was registered at the level of bulk CTL cytotoxicity and may result from enhancement of APC function and/or direct effects on T cell responders. We are currently evaluating the impact of these transfected cytokines on the actual frequency of CTL responders using Ag-specific cytokine enzyme-linked immunospot assays. Our results are consistent with observations in murine DNA vaccine studies in vivo in which the coadministration of GM-CSF or IL-12 genes also resulted in an augmentation of Ag-specific CTL responses (6–8). The result that cotransfection of IFN-α also promotes the induction of CTL responses has not been reported to date. IFN-α, like IL-12, is produced by DC in response to infectious agents (43–46). Both IL-12 and IFN-α enhance the generation and increase the cytotoxicity of NK cells and CD8$^+$ CTL. Importantly, IL-12 promotes a Th1-like phenotype (characterized by high IFN-γ production) in the Ag-specific differentiation of

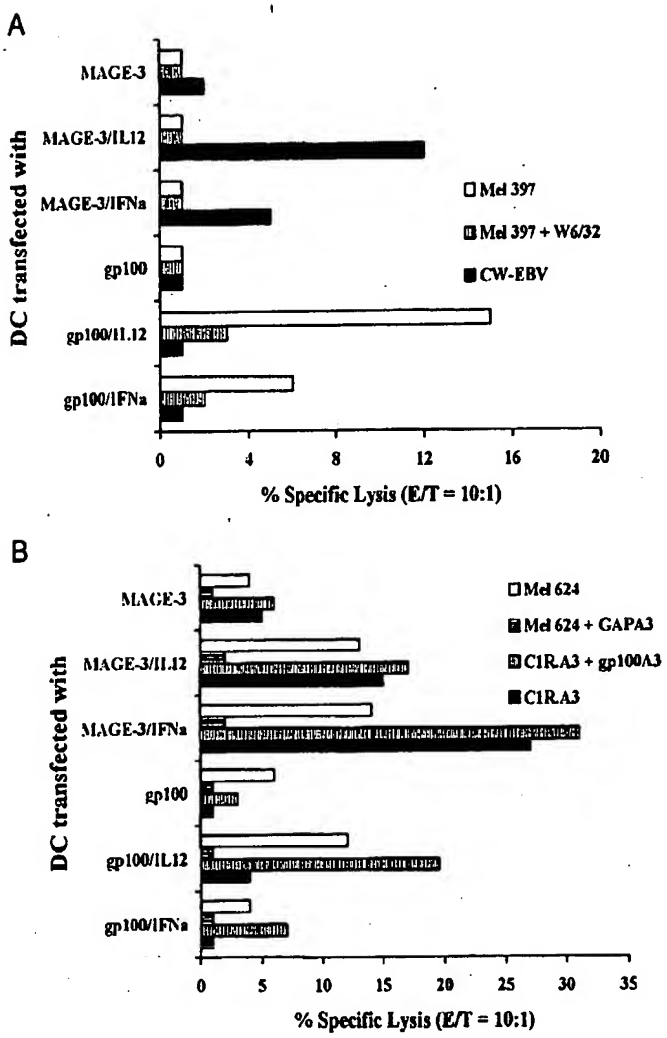

FIGURE 5. Identification of novel MHC-restricted CTL reactivities against pMel-17/gp100 and MAGE-3 using in vitro gene vaccination. HLA-A1$^+$, -A3$^+$, -B8$^+$, -B51$^+$, -Cw3$^+$ cultured DC were transfected with MAGE-3 cDNA or pMel-17/gp100 cDNA with or without cytokine cDNA as indicated. Autologous CTL were induced, as outlined above, with bulk responder T cell evaluated for cytolytic reactivity on day 21 (7 days after the third stimulation) against Mel-397 and CW-EBV and for inhibition of this reactivity by mAb W6/32. pMel-17/gp100-reactive and W6/32-inhibitable CTL capable of lysing the Mel-397 (HLA-A1 and -B8 common alleles with responder) but not CW-EBV were noted (*A*), particularly if IL-12 or IFN-α cDNA were applied in the primary stimulation. In *B*, Mel-624-reactive CTL restricted by HLA-A3 were identified in both the MAGE-3 and gp100 cultures. A significant portion of the HLA-A3-restricted CTL reactivity appeared to be directed against the $gp100_{17-25}$ (gp100A3) epitope recently identified by Skipper et al. (32). The data are representative of two such experiments performed.

naive CD4$^+$ T cells. This has also been demonstrated for IFN-α (47, 48). On a molecular level, IFN-α appears to induce the expression of the IL-12R β2 subunit, which is selectively expressed by human Th1 cells (49). This may suggest that IFN-α-mediated enhancement is dependent upon constitutive secretion of IL-12 by activated DC, a hypothesis that we are currently evaluating.

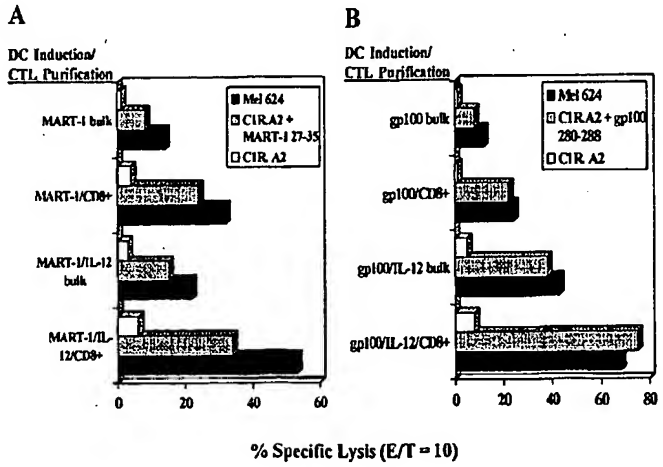

FIGURE 6. CD4$^+$ T cell depletion enriches for antitumor-reactive CTL induced with genetically modified autologous DC. Bulk HLA-A2$^+$ T cell cultures were stimulated with autologous DC transfected with either MART-1 with or without IL-12 cDNA (*A*) or gp100 cDNA with or without IL-12 cDNA (*B*). After three weekly stimulations, CTL were evaluated for their ability to lyse Mel-624 (HLA-A2$^+$, MART-1$^+$, gp100$^+$) cells, C1R.A2 (HLA-A2$^+$, MART-1$^-$) cells, or C1R.A2 cells pulsed with the indicated peptides in standard 4-h chromium release assays. The enhanced cytolytic activity noted in these two experiments is reflective of three CD4$^+$ T cell depletions evaluated.

A major strength of gene-based vaccines is that their application does not require prior knowledge of the patient HLA haplotype or of specific T cell epitopes, such as those implemented in peptide-based approaches. The expression of entire melanoma Ags within the APC allows for the potential concurrent processing and presentation of multiple, clinically important, but as yet undefined MHC-restricted epitopes. Our results suggest that a given melanoma-associated Ag may be presented in an immunogenic format by transfected DC, resulting in the induction of anti-melanoma-reactive CTL restricted by more than one HLA class I allele. Specifically, MART-1/Melan-A, pMel-17/gp100, tyrosinase, MAGE-1, and MAGE-3 appear to contain peptide epitopes presented by the HLA-A1, -A2, and -A3 alleles. Given the combined frequency of these alleles in the melanoma patient population (~70%), gene-based approaches employing such tumor Ag cDNAs could be theoretically applicable to the majority of patients. Further, as noted previously, the diversification of the induced T cell immune response to multiple HLA restriction elements reduces the likelihood of HLA allele loss variants escaping therapy-induced immunity.

Another potential advantage of DNA vaccines may be the stimulation of Ag-specific MHC class II-restricted T cell responses. Preliminary evidence suggests that this may be particularly true for melanosome-targeted Ags such as pMel-17/gp100, tyrosinase, or the tyrosinase-related proteins, which naturally localize into the endosomal compartments (50), giving rise to peptides presented in association with MHC class II molecules. A single report has described $CD4^+$ T cell-defined epitopes in the melanoma setting that derive from the tyrosinase gene product (51). Tumor Ags that normally do not traffic into endosomal compartments could be engineered to do so by addition of appropriate signal sequences (52). Vaccine-induced, Ag-specific $CD4^+$ T cell reactivity may promote durable therapeutic immune responses, given the recent finding that Ag-specific $CD4^+$ T cell responses are required for long term maintenance of CTL (53, 54).

In summary, we provide evidence that molecular engineering of host DC to express five different melanoma-associated Ags yields a potent immunogen, capable of promoting Ag-specific CTL in vitro, and that this approach further benefits by inclusion of transfected Th1-biasing cytokines, such as IL-12 or IFN-α. The clinical application may involve transdermal tumor Ag cDNA vaccination using the gene gun (transfection of resident DC/Langerhans cells in the skin) or the ex vivo engineering of DC followed by adoptive transfer. Melanoma patients who are not immunosuppressed, especially those with minimal residual disease but at high risk for recurrence, may particularly benefit from such approaches.

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